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Truffle mycorrhization in a pecan orchard

Micorrização de trufas em um pomar de nogueira-pecã

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Highlights

Mycorrhization of Tuber spp is maintained in an under Brazilian subtropical conditions.
Ectomycorrhizal colonization of truffles reduced under orchard conditions.
The colonization of other ectomycorrhizae may reduce association with species of the genus Tuber.

Abstract

The ectomycorrhizal association of *Tuber* spp. in orchards is essential for truffle production. In this sense, this study aimed to evaluate the ectomycorrhizal association of *Tuber aestivum* Vittad. and *Tuber brumale* Vittad. in pecan after five years of cultivation in an orchard. The experiment was conducted in Santa Maria – Rio Grande do Sul, Brazil, in a pecan orchard planted with seedlings inoculated with these two species of truffles. The orchard was characterized in terms of soil physical and chemical properties and soil type. Initially non-inoculated plants, plants inoculated with *Tuber aestivum* (TA), and plants inoculated with *Tuber brumale* (TB) were evaluated for the percentage (%) of roots colonized by *Tuber* spp., other ectomycorrhizae, and non-mycorrhizal roots. A reduction in the colonization of both truffle species was observed five years after transplanting the seedlings to the orchard but they have the potential to maintain the ectomycorrhizal association with pecan seedlings.

Key words: *Carya illinoinensis.* Ectomycorrhiza. Truffle. *Tuber.*

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Resumo

Em pomares, a presença da associação ectomicorrízica de *Tuber* spp. é fundamental para a produção de trufas. Portanto, este estudo objetivou avaliar a associação ectomicorrízica de *Tuber aestivum* Vittad e *Tuber brumale* Vittad em nogueira-pecã, após cinco anos de cultivo em pomar. O experimento foi conduzido em Santa Maria – Rio Grande do Sul, Brasil, em um pomar de nogueira-pecã implantado com mudas inoculadas com estas duas espécies de trufas. O solo do pomar foi caracterizado para as propriedades físicas e químicas do solo e o quanto ao tipo de solo. As plantas não inoculadas, inoculadas com *Tuber aestivum* (TA) e inoculadas com *Tuber brumale* (TB) foram avaliadas quanto ao percentual (%) de raízes colonizadas por *Tuber* spp., outras ectomicorrizas e raízes não micorrizadas. Após cinco anos do transplante das mudas ao pomar, houve redução da colonização de ambas as espécies de trufas, porém estas apresentam potencial de manter a associação ectomicorrízica com mudas de nogueira-pecã.

Palavras-chave: *Carya illinoinensis.* Ectomicorriza. Trufa. *Tuber.*

Introduction

Pecan (*Carya illinoinensis* [Wangenh. K. Koch]) cultivation presents an excellent cost-benefit ratio and can be exploited economically over a long period and with low production costs (Martins et al., 2017; Fronza et al., 2018). The first pecan plantings in Brazil occurred in 1870 in the municipality of Americana (SP) after the introduction of seedlings from the United States and began to be cultivated commercially following government incentives in the 1970s (Fronza et al., 2018). Currently, it is most common in the states of Rio Grande do Sul, São Paulo, and Minas Gerais (Fronza et al., 2016). For instance, pecan production in the state of Rio Grande do Sul involves about 1100 families (Martins et al., 2018), which account for around 70% of national production, in addition to having 90% of the fruit processing industries (Rubin, 2023). The Intituto Brasileiro de Pecanicultura [IBPecan] (2023) predicted the production of 7 thousand tons in 2023, cultivated on approximately 6 thousand hectares, with an export value ranging from US$ 2.89/kg to US$ 4.30/kg. Despite already achieving considerable production levels, Brazil has significant potential for further expanding pecan cultivation. This potential stems not only from its profitability but also from promising opportunities for growth within both the domestic consumer market and the export sector.

In addition to its economic relevance in nut production, pecan cultivation has ecological advantages through the association of fungi with plant roots (Sulzbacher et al., 2019). Symbiosis with ectomycorrhizal fungi stimulates host growth by increasing the nutrient absorption area and protecting the plant from root pathogens (Huey et al., 2020). Furthermore, the symbiosis between pecan and ectomycorrhizal fungi can boost another economic activity of great profitability for farmers, truffle farming (Benucci et al., 2011). In 2016, the discovery of truffles (*Tuber floridanum* A. Grupe, Sulzbacher & M.E. Smith) in a pecan orchard in the state of Rio Grande do Sul (Grupe et al., 2018; Sulzbacher et al., 2019) revealed the possibility of co-producing truffles and nuts in Brazil. Truffles are hypogeous fungi with high gastronomic and commercial value due to the organoleptic properties of their aroma (Bonito et al., 2009; Culleré et al., 2013; Grupe et al., 2018). The Brazilian truffle (Sapucay) has a unique and pleasant aroma, incomparable to other commercial species of the same genus (Freiberg et al., 2023). This fungus produce ripened fruits in the summer, from October to mid-February, and is harvested in the area under the projection of the plant crown, where the truffle can be found in a depth of 5 to 20 cm of soil, depending on the distribution of the roots in the soil profile (Freiberg et al., 2023).

One of the steps for truffle production involves obtaining well-developed seedlings with adequate levels of colonization of the truffle species of interest. In this sense, Freiberg et al. (2021) adapted the seedling mycorrhization protocol for Brazilian subtropical conditions and obtained high levels of colonization in pecan roots inoculated with two truffle species (*Tuber aestivum* and *Tuber brumale*). However, these results are restricted to the nursery environment, with the need to evaluate the performance of inoculated seedlings under orchard conditions. Therefore, this study aimed to evaluate the ectomycorrhizal association of *T. aestivum* and *T. brumale* in pecan after five years of cultivation in an orchard.

Material and Methods

History and characterization of the pecan orchard

The study was conducted in a pecan orchard in the experimental area of the Polytechnic College of the Federal University of Santa Maria, in the municipality of Santa Maria, located in the physiographic region of the Central Depression of Rio Grande do Sul (29°42′52.7′′S; 53°44′17.8′′W; approximate altitude 125 meters). The regional climate is Cfa, according to the Köppen classification, which corresponds to the humid subtropical climate, with a mean annual precipitation of 1620 mm, mean temperature of −3 °C in the coldest month and 22 °C in the hottest month (Alvares et al., 2013). The orchard soil is characterized as an Argissolo Bruno-Acinzentado, whose main characteristic is the diagnostic B textural horizon with a predominance of grayish colors with a hue of 7.5YR or more yellow, value higher than or equal to 5, and chroma lower than or equal to 4 (H. G. Santos et al., 2018). The soil profiles of the experimental area are characterized by the sequence of horizons AP–A–A1–Bt1–Bt2 (Figure 1 a,b,c), with a silt loam texture in the A horizon, silty clay loam texture in the A1 horizon, and clay increment in the B horizons. The A horizon has color 10YR 3/2, A1 7.5YR 3/2, Bt1 10YR 5/4, and Bt2 a grayish matrix (10YR 4/1) and concretions in color 2.5YR 5/8.

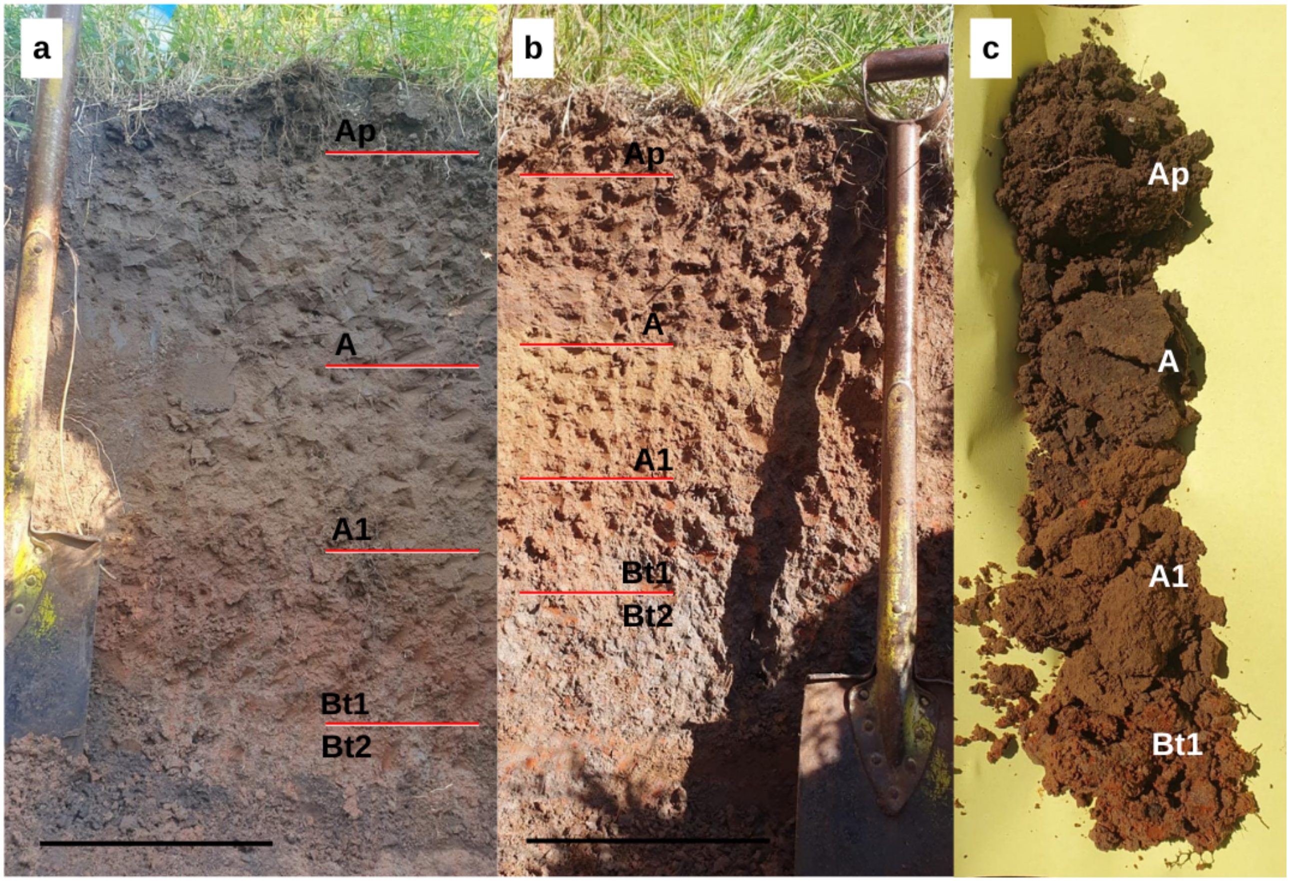

Figure 1. Soil profile in the pecan orchard (a, b), highlighting the color differences between horizons (c) (30 cm).

The pecan orchard contained 25 mycorrhized seedlings arranged in six rows with an initial spacing of 7 x 8 m. Fifteen mycorrhized seedlings with *T. brumale* and 12 mycorrhized seedlings with *T. aestivum* were transplanted in 2017, totaling 25 pecan seedlings (Barton and Imported cultivars). The orchard was densified with new seedlings in 2020 and 2021, reducing the spacing to 3.5 x 8 m, aiming to obtain smaller trees with standardized sizes. Currently, 95 plants (non-inoculated pecan – pre-established, pecan inoculated with *Tuber* spp., and new pecan – seedlings from densification) make up the orchard (Figure 2).

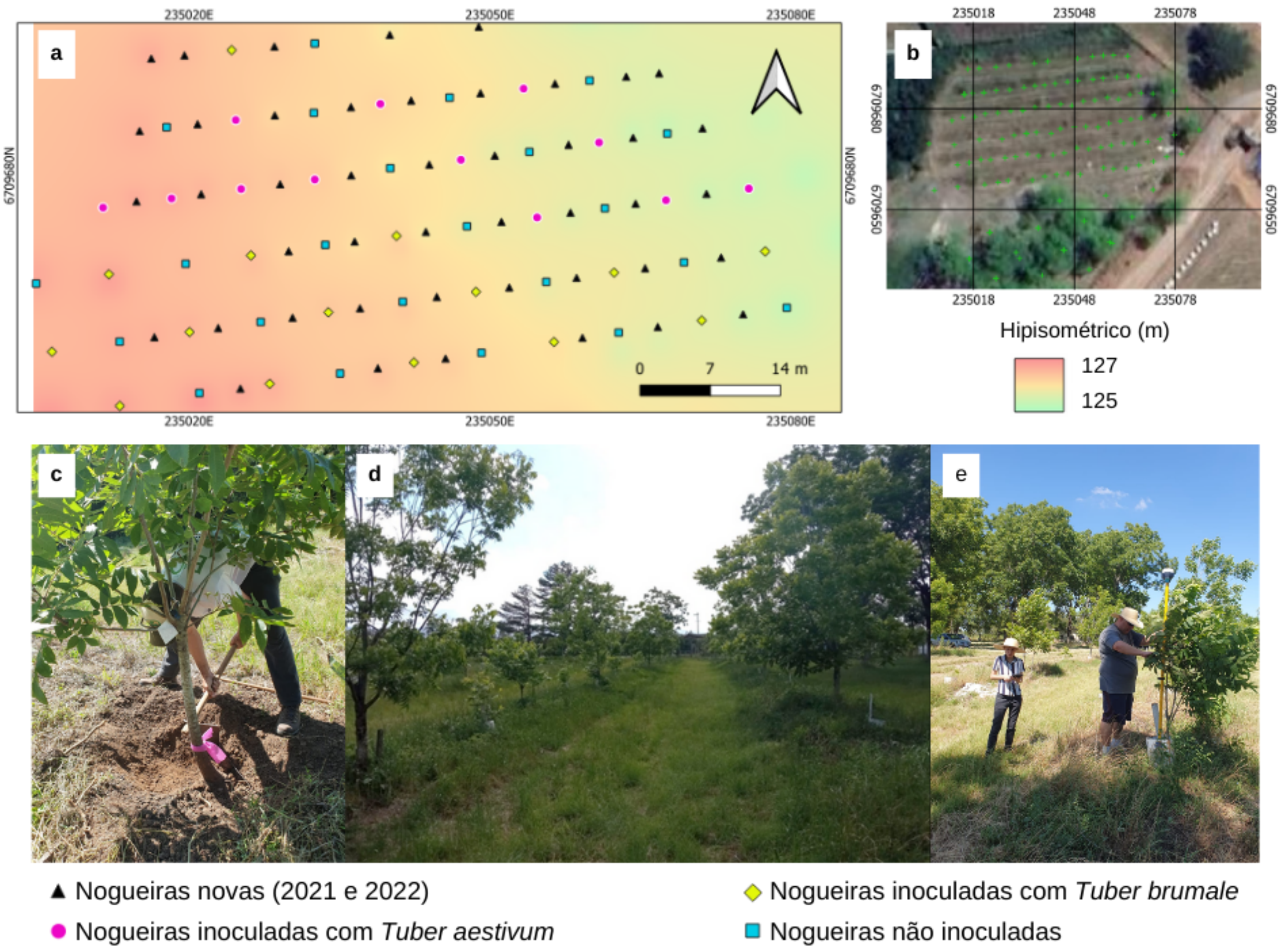

Figure 2. Location map of the experimental area and distribution of pecan plants in the UFSM orchard, Santa Maria/RS (a) (b). Photos of the study area (c) (d) (e).
Hypsometric (m); New pecan plants (2021 and 2022); Pecan plants inoculated with Tuber aestivum; Pecan plants inoculated with *Tuber brumale*; Non-inoculated pecan plants.

Soil physical and chemical properties

Soil subsamples (140 g) were collected at a depth of 0–20 cm from ten plants of each treatment, totaling 30 subsamples. The subsamples were homogenized, forming a composite sample for each treatment: plants inoculated with *T. aestivum* (TA), plants inoculated with *T. brumale* (TB), and non-inoculated plants (NI). A total of 150 g of soil volume was taken from this composite sample to evaluate the soil chemical parameters (Teixeira et al., 2017), including phosphorus (Mehlich-1 extractor solution), potassium (Mehlich-1), exchangeable cations – calcium, magnesium, aluminum (1 mol L^{-1} KCl), copper and zinc (0.1 M HCl), boron (hot water), sulfur (calcium phosphate), clay (NaOH), organic matter (sulfochromic solution), and pH (in water 1:1) (Table 1). The characterization of soil physical properties (Teixeira et al., 2017) was carried out in two trenches at depths of 0 to 5 cm, 5 to 15 cm, 15 to 30 cm, and 30 to 50 cm, with four repetitions per depth, to determine soil density (volumetric cylinder method), total porosity (water saturation), and microporosity and macroporosity (tension table) (Table 2).

Table 1
Characterization of soil chemical properties in the pecan orchard inoculated with *Tuber aestivum* (TA) and *Tuber brumale* (TB) and non-inoculated seedlings (NI)

Chemical parameter	Treatment		
	TA	TB	NI
pH (1:1 water)	6.3	6.5	6.8
Ca (mg/dm³)	1303	1783	2004
Mg (mg/dm³)	462	535	851
Al (mg/dm³)	0	0	0
Al saturation (%)	0	0	0
Base saturation (%)	84.1	86.6	91.5
SMP index	6.5	6.6	6.8
%OM (m/v)	4.7	4.8	6.1
%Clay (m/v)	19	19	8
Texture	4	4	4
S (mg/dm³)	32.8	28.1	19.7
P-Mehlich (mg/dm³)	157.2	161.8	304
K (mg/dm³)	268	360	348
Cu (mg/dm³)	1.44	1.02	0.84
Zn (mg/dm³)	9.27	9.23	15.1
B (mg/dm³)	1.61	1.47	1.81
Ca/Mg	2.2	2	1.4
(Ca+Mg)/k	18	14.4	19
K/(Ca+Mg)$^{1/2}$	0.195	0.253	0.216

Table 2
Soil physical properties of the pecan orchard. Mean percentage of soil density (Ds), total porosity (Pt), macropores (Ma), and micropores (Mi) for each collection depth, followed by the coefficient of variation (CV) of each mean

Depth	Ds ($g\ cm^{-3}$)	CV (%)	Pt ($cm^3\ cm^{-3}$)	CV (%)	Ma ($cm^3\ cm^{-3}$)	CV (%)	Mi ($cm^3\ cm^{-3}$)	CV (%)
0-5 cm	1.2477	5.2	0.4803	7.0	0.1910	14.3	0.2893	12.0
5-15 cm	1.3882	3.4	0.4289	6.7	0.1517	14.0	0.2772	4.8
15-30 cm	1.4055	4.8	0.4142	4.7	0.1343	20.3	0.2798	5.5
30-50 cm	1.5167	2.1	0.3922	5.2	0.0738	23.5	0.3184	2.6

Soil density (Ds), total porosity (Pt), macroporosity (Ma), and microporosity (Mi). Coefficient of variation (CV).

Assessment of ectomycorrhizal colonization

Ten trees from each treatment (TA and TB) were randomly selected for root collection, approximately one meter from the stem, in the north, northeast, and northwest directions, where there is a higher probability of finding ectomycorrhizal fungi (Bonito et al., 2009). However, the methodology was adapted to collect roots in different directions at a distance of 0.5m from the stem due to the age of the orchard and the drought. The soil was gently excavated to avoid damaging the root system, and the fine roots were collected, packed in polypropylene bags, transported in a Styrofoam box, and stored at approximately 4 °C until processing. All samples were processed within 10 days.

The collected roots were immersed in water until the soil residues dissolved to evaluate ectomycorrhizal colonization. Afterward, they were gently washed in a 1-mm opening sieve and placed in Petri dishes with water. The percentage of roots mycorrhized with *Tuber* spp., roots mycorrhized with other ectomycorrhizae, and non-mycorrhized roots were counted under a stereomicroscope (Brundrett et al., 1996). Characteristic structures of *Tuber* spp. (mantle, Hartig net, extraradicular mycelium) were observed under a microscope, as described by Agerer (1991, 2012).

Data analysis

Ectomycorrhizal colonization results were expressed as mean percentage values followed by standard deviation (n = 12). The values of chemical properties were expressed as absolute values (n = 1) and physical properties as mean values (n = 4), followed by the coefficient of variation.

Results and Discussion

The association of *T. aestivum* and *T. brumale* remained in pecan seedlings after transplantation to the orchard. Characteristic structures of *T. aestivum* and *T. brumale*, such as yellowish-brown monopodial-pyramidal branching and dense mycelium, were observed (Figure 3). *T. aestivum* presented small cystidia located at the apex of the mantle (Figure 3a, b), while *T. brumale* presented cystidia with the same morphology but located laterally (Figure 3c). The structure of the pseudoparenchymatous mantle, composed of polygonal cells in both species, with rounded angles in *T. aestivum* and oval in *T. brumale*, was observed with a microscope. There was adaptation of *Tuber* spp. to the edaphoclimatic conditions of the region despite the low values of colonization by *T. aestivum* and *T. brumale* in the pecan orchard. The mycorrhization rate of the species of interest in the field was 56.7% and 72.4% lower than the mean colonization rate obtained by Freiberg et al. (2021) for *T. aestivum* and *T. brumale*, respectively, under nursery conditions. Likewise, D. O. Santos (2021) also obtained a high colonization rate of *T. aestivum* and *T. floridanum* in pecan seedlings (approximately 90%) under greenhouse conditions, contrary to Morcillo (2007), who obtained 53.84% for *T. brumale* in a hazelnut orchard (*Corylus avellana* L.).

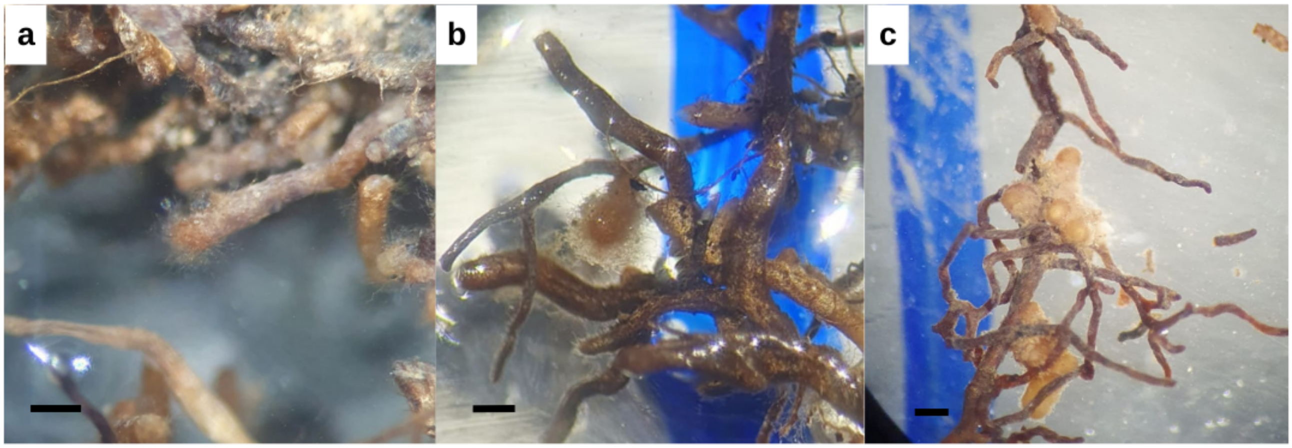

Figure 3. Mycorrhization of *Tuber aestivum* (a) (b) and *T. brumale* (c) in pecan roots observed under a stereoscopic microscope.

Truffle production in an intercropping of tree species (*Carpinus betulus, Quercus pubescens, Corylus avellana, Pinus nigra,* and *Tilia cordata*) in France started after five years of planting, reaching its productive peak after 13 years, with approximately 320 kg/ha (Todesco et al., 2019). The first fruiting in a hazelnut orchard in Burgundy inoculated with *T. melanosporum* and *T. aestivum* occurred four years after planting when 71 out of the 98 planted trees produced at least one truffle (Molinier et al., 2013). Therefore, truffle fruiting requires at least four years to start production, depending on the structural soil conditions, the amount of *Tuber* spp. mycelium, soil temperature conditions, and water stress (Todesco et al., 2019). In Brazil, the presence of *T. floridanum* (Sulzbacher et al., 2019; Freiberg et al., 2023) highlights the possibility of truffle farming under Brazilian subtropical conditions. However, new studies must be carried out to investigate the presence of other truffles introduced in Brazil and the factors that affect their fruiting and production. Collections of *T. floridanum* from pecan plants point to a higher presence of truffles in irrigated, well-drained, shaded areas with a higher supply of organic matter (litter and nutrients), factors also observed by Bonito et al. (2013) and Culleré et al. (2010) with other truffle species.

One of the main agronomic characteristics of successful truffle production is good soil structure, enabling the circulation of water and gases throughout the profile (Chevalier & Sourzart, 2012b). This recommendation is also valid for pecan cultivation (Martins et al., 2017), which highlights the common abiotic needs of the host and the fungus. Pecan requires a large amount of water, developing best in soils with a clay loam texture (Fronza et al., 2018). On the other hand, Neossolos Flúvicos and Litólicos (Entisols), characterized by shallow depth and high pH values, provide better conditions for truffle development (Chevalier & Frochot, 1990; Chevalier, 2012a). Regarding soil chemical properties, the ideal development of pecan trees occurs at pH 6.0 (Comissão de Química e Fertilidade do Solo [CQFS], 2016; Martins et al., 2017). In general, truffles require more alkaline soil conditions, with pH ranging from 6.6 to 8.4 for *T. aestivum* (Chevalier & Sourzart, 2012b) and 7.2 to 8.7 for *T. melanosporum* (Zambonelli et al., 2015). Soil density values were observed within the normal range, with a limit of up to 1.75 mg m^{-3} (Table 3). However, poor drainage, a characteristic of flat soils and the area under study, may have harmed both the development of pecan trees and the maintenance of the ectomycorrhizal association and truffle fruiting. According to Fischer et al. (2017), truffle productive sites are slightly sloped and have good drainage. In addition to these criteria, the selection of quality hosts and adequate management of pests and diseases are also factors for the productive success of truffles.

Another important factor for successful truffle production is root contamination by other ectomycorrhizae, replacing or preventing the development of the ectomycorrhizae of interest (Benucci et al., 2011; Bonito et al., 2011). The seedlings used to establish the orchard were obtained from a nursery, with an average of 70% of the roots colonized by *T. aestivum* and 88% by *T. brumale.* In this sense, root colonization by other ectomycorrhizae was higher than 40%, with non-inoculated plants showing colonization of 60.58% (Table 3). There is a possibility that the seedlings were contaminated by *Hebeloma* sp., *Scleroderma* sp., and *Tomentella* sp., as these fungi were present in the nursery on the roots of non-inoculated seedlings (Freiberg et al., 2021). Furthermore, the pre-existence of pecan plants in the orchard and its surroundings can also contribute to the accidental introduction of undesirable spores. Pecan orchards in the state of Rio Grande do Sul have the occurrence of reproductive structures of other ectomycorrhizal fungi such as *Astraeus* sp., *Hymenogaster* sp., *Inocybe* sp., and *Russula* sp. (Sulzbacher et al., 2019), which may also have spores dispersed and contaminating orchards.

Table 3

Percentage (%) of roots colonized by *Tuber* spp. (*T. aestivum* and *T. brumale*) and other ectomycorrhizae and non-mycorrhized roots in a pecan orchard after eight years of inoculation with European truffle species

Treatment	Other ectomycorrhizae (%)	*Tuber* sp. (%)	Non-mycorrhized (%)
Non-inoculated	$61.3^{\pm 9.51}$	$0.0^{\pm 0.00}$	$38.8^{\pm 9.51}$
Inoculated by *Tuber aestivum* (TA)	$42.3^{\pm 11.96}$	$13.3^{\pm 11.59}$	$43.8^{\pm 10.95}$
Inoculated by *Tuber brumale* (TB)	$42.4^{\pm 13.16}$	$15.6^{\pm 10.73}$	$42.2^{\pm 7.72}$

Means followed by standard deviation. Number of observations per treatment = 12.

Conclusion

Tuber aestivum and *Tuber brumale* have the potential to maintain the ectomycorrhizal association with pecan seedlings after transplanting to the orchard despite the reduction in its colonization. Therefore, new investigations should be conducted to elucidate the biotic and abiotic factors that influence the maintenance of the ectomycorrhizal association of truffles under Brazilian subtropical conditions.

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